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Bioequivalence evaluation of two D-limonene capsule formulations in healthy Chinese volunteers

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A randomized, three-period crossover study was conducted in 24 healthy Chinese male volunteers to compare the bioavailability of two brands of D-limonene (0.3 ml) capsules, and determine the plasma concentration of endogenous D-limonene in food-controlled non-treated humans. The three kinds of treatments were administration of the reference formulation, administration of the test, and non-administration. The plasma samples were analyzed by a validated GC-MS method after liquid-liquid extraction. The pharmacokinetic parameters AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, and $t_{1/2}$ were determined from the concentration-time profiles for both formulations and were compared statistically to evaluate bioequivalence between the two brands. The analysis of variance (ANOVA) did not show any significant difference between the two formulations and 90% confidence intervals fell within an acceptable range for bioequivalence. Besides, for the food-controlled non-treated volunteers, their plasma concentrations of D-limonene were detectable and kept relatively steady (2.94 ± 1.38 ng/ml) within the sample collection period. Based on the statistical analysis, it was concluded that the two D-limonene capsule formulations were bioequivalent.

1. Introduction

D-Limonene (1-mentha-4-isopropyl-cyclohexene), a naturally occurring monocyclic monoterpene found in various fruits and vegetables, is widely used as an ingredient in spices, beverages, and cosmetics. It was reported that large doses of D-limonene have anticancer activity, while low doses of D-limonene can be used to treat gallstones, cholecystits, angiocholitis, etc (Wang 2005). The pharmacokinetics of D-limonene in Sprague-Dawley rats following orally administration show a biphasic profile with a mean terminal $t_{1/2}$ of 337 min and an oral bioavailability of 43.0% (Chen et al. 1998). Besides, D-limonene is an endogenous substance in rats' plasma, and might well come from the diet (Chen et al. 1998). Though several pharmacokinetic studies for D-limonene have been reported (Chen et al. 1998; Wang et al. 2007; Vigushin et al. 1998; Falk et al. 1993), none of them have focused on bioequivalence between two market brands, or the determination of endogenous D-limonene in healthy human subjects.

The purpose of this study was to determine the bioequivalence of a new capsule formulation of D-limonene produced by Sichuan Changwei Pharmaceutical Co., LTD (China), in comparison with a another capsule produced by Sichuan Huaxin Pharmaceutical Co., LTD (China), and reveal the plasma level of endogenous D-limonene in food-controlled non-treated human.

2. Investigations and results

All volunteers who started the study continued to the end and were discharged in good health. The demographic and mean health parameters of all participants are summarized in Table 1; as can be observed, volunteers formed a homogeneous population in terms of age, weight, height and BMI.

The analytical method was validated in compliance with the guidelines of FDA (FDA 2001b). The lower limit of quantification was 0.57 ng · ml^{-1}, far lower than that reported before (Chen et al. 1998; Wang et al. 2007; Vigushin et al. 1998; Falk et al. 1993). Calibration standards demonstrated acceptable linearity ($r = 0.9990$) within a concentration range of $0.57 \sim 456.4$ ng · ml^{-1} ($R = 0.025307C + 0.00219$). The absolute recovery was 102.69%, while the relative recovery ranged from 90.11% to 100.96%. The intra-day and inter-day precisions ranged from 0.26% to 3.73% and

Table 1: Demographic and health parameters of all 24 healthy Chinese male volunteers considered in the bioequivalence study

	Age (year)	Height (m)	Weight (kg)	BMI (kg · m^{-2})	Breath rate (min^{-1})	Heart rate (min^{-1})	Temp. (°)	Blood pressure (mmHg)
Mean	21.54	174.54	65.63	21.62	19.02	74.42	36.24	118.66/75.25
SD	0.98	6.60	6.08	1.21	0.52	7.23	0.11	4.70/5.14

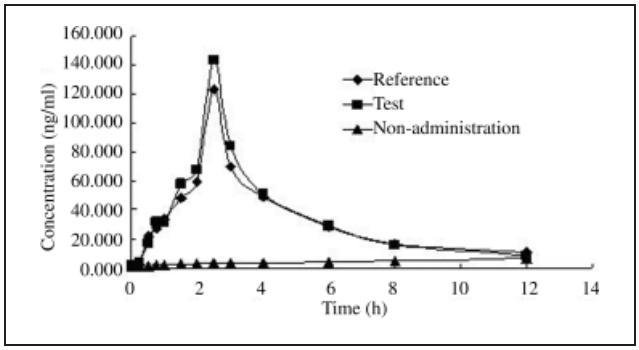

Fig. 1: Mean plasma concentration–time curve of D-limonene in 24 food-controlled healthy Chinese male volunteers after administration of reference formulation, administration of test formulation, and non-administration

1.68% to 8.15%, respectively. The stability study showed that D-limonene was stable in plasma samples for at least 24 h at room temperature (approximately 20 °C); three freeze-and-thaw cycles; 13 days at −20 °C. The method used in this study was found to be reliable, accurate, sensitive and rapid for detecting plasma levels of D-limonene.

Mean plasma concentration-time profiles of D-limonene for the three treatments (administration of the reference formulation, administration of the test, and non-administration) are shown in Fig. 1. The intrasubject and intersubject variability for C_{max} and $AUC_{0-\infty}$ are shown in Fig. 2. Table 2 shows the pharmacokinetic parameters of D-limonene for the two formulations. Analysis of variance (ANOVA) for the PK parameters was performed after natural log-transformation of the data, and showed no statistically significant difference between the two formulations, with a value of $p > 0.05$. As can be seen in Table 2, 90% CI for all compared pharmacokinetic parameters (ratios of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max}) were contained in the 80%–125% interval; the two one-sided tests for all analyzed parameters showed no significant difference. The relative bioavailability of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were 99.0%, 99.3% and 100.6%, respectively. Besides, non-parametric statistical tests of t_{max} revealed no statistically significant difference between the test and reference formulation. Moreover, the test and reference formulation exhibited similar values of $t_{1/2}$ and the percentage of AUC extrapolated to infinity was similar for the test and reference formulation.

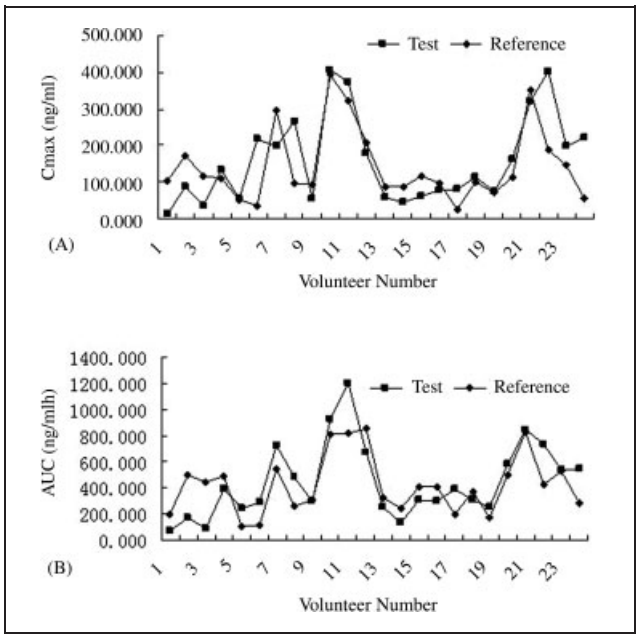

Fig. 2: Intrasubject and intersubject variability in the estimation of plasma pharmacokinetic parameters of D-limonene in 24 food-controlled healthy Chinese male volunteers after single oral administration of test and reference formulations (A, C_{max}; B, $AUC_{0-\infty}$)

3. Discussion

It was reported that the maximum tolerated dose was $8\ g \cdot m^{-2}$ per day in advanced cancer patients (Vigushin et al. 1998). As unexpected incidents that could have influenced the outcome of the study did not occur during the study, it can be concluded that D-limonene was well tolerated.

Polygeline injection is a plasma expander, which contains components (such as peptides) similar to human plasma, but no D-limonene. As our pretest (data not shown) revealed there was D-limonene in human blank plasma, all calibration standards and quality control standards were prepared with polygeline injection instead of blank plasma, to establish a method that can determine the plasma concentration of endogenous D-limonene. Besides, pretests demonstrated that *n*-hexane might dissolve plastic, and the solute could affect the determination of D-limonene, and that D-limonene was easily adsorbed on the wall of glass tubes. Therefore, in this study, silanized glass tubes were used instead of plastic tubes.

Figure 1 indicates that the mean plasma concentration profiles of the two formulations were similar. Besides, in food-controlled non-treated volunteers, although all of them had fasted overnight, plasma concentrations of D-limonene were still detectable. Surprisingly, the concentration was relatively steady (2.94 ± 1.38 ng/ml) within the sample collection period (12 h). It was reported that D-limonene can be detected in rat's blank plasma (500–600 ng/ml), and that the concentration may reduce signifi-

Table 2: Pharmacokinetic parameters of two D-limonene capsule formulations in 24 food-controlled healthy Chinese male volunteers after single oral administration

Pharmacokinetic parameter	Test	Reference	90% CI[a] (80%–125%)	Two one-sided tests[a] ($p < 0.05$)	
				Low	High
AUC_{0-t} (ng/mlh)	420.812 ± 279.795	394.030 ± 212.160	84.8%–115.5%	0.013	0.007
$AUC_{0-\infty}$ (ng/mlh)	449.855 ± 285.740	423.505 ± 225.206	80.7%–122.3%	0.044	0.035
C_{max} (ng/ml)	159.573 ± 120.533	143.535 ± 100.817	81.3%–124.4%	0.003	0.004
t_{max} (h)	2.083 ± 0.615	2.781 ± 2.269			
$t_{1/2}$ (h)	2.859 ± 1.162	2.838 ± 0.947			
$AUC_{0-t}/AUC_{0-\infty}$	0.928 ± 0.061	0.930 ± 0.049			

Values are given as mean ± standard error

[a] Statistics were applied on natural logarithm-transformed data; n = 24

cantly (nearly tenfold) if the blood was obtained from a rat after overnight fasting (Chen et al. 1998). These results implied that D-limonene detected in the plasma might not only come from food or medication, but also be endogenously biosynthesized.

Table 2 revealed that the AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} in this study were relatively bigger than that observed in another study at a same dose (275.8 ± 110.1 ng/ml h, 311.9 ± 108.8 ng/ml h, and 93.6 ± 64.8 ng/ml for AUC_{0-t}, $AUC_{0-\infty}$, and C_{max}, respectively) (Wang et al. 2007). These differences might well result from the better absorption of D-limonene from the products in this study. Besides, the $t_{1/2}$ is smaller than that reported before (Wang et al. 2007). Furthermore, the estimate value of AUC_{0-t} being $> 90\%$ of the estimate value of $AUC_{0-\infty}$ implied that the sampling scheme was sufficiently long to ensure an adequate description of the absorption phase. The statistical analysis of AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, and t_{max} between the test and reference formulations (both 0.3 ml D-limonene capsules) indicated no significant differences in any pharmacokinetic parameter. Therefore, it can be established that Ningmengxijiaonang, produced by Sichuan Changwei Pharmaceutical Co., LTD, China, is bioequivalent to Lidanqing produced by Sichuan Huaxin Pharmaceutical Co., LTD, China, and that both formulations can be considered equally effective in therapy.

4. Experimental

4.1. Study products

Test product: Ningmengxijiaonang- D-limonene 0.1 ml capsule, Batch No.: 20060901; Expiration date 09/2008, Manufacturer: Sichuan Changwei Pharmaceutical Co., LTD, China.

Reference product: Lidanqing- D-limonene 0.1 ml capsule, Batch No.: 20060802; Expiration date 08/2008, Manufacturer: Sichuan Huaxin Pharmaceutical Co., LTD, China.

4.2. Study design

Twenty-four healthy Chinese male volunteers participated in this comparative study at the Clinical Trail Base in West China Women's & Children's Hospital under the following inclusion criteria: age between 18 and 27 years; non-smokers and non-drinkers or having quit smoking and drinking 72 h before the beginning of the study; with a body mass index (BMI) between 19 and 24; a normal clinical history; thorax radiography and electrocardiogram without abnormalities; normal values in laboratory tests (haematology, blood biochemistry, hepatic function and urine analysis) and negative results for HIV and hepatitis types B. For 2 weeks prior to and during the study period, all subjects were served with food had been fully inspected that do no contain D-limonene, and instructed to abstain from taking any food that may contain D-limonene (such as lemon, orange, beverage, etc) or drug including over-the-counter (OTC). They were informed about the aim and risks of the study by the clinical investigator, based on which they signed a written informed consent statement before entering the study. The study protocol was reviewed and approved by the Ethics Board of West China Medical Center, Sichuan University, China.

4.3. Drug administration and sample collection

The study was designed as a single dose, randomized, three-treatment, and three-period crossover. The three kinds of treatments were administration of the reference formulation (0.3 ml D-limonene), administration of the test (0.3 ml D-limonene), and non-administration, respectively. In the morning of phase I, after an overnight fast (12 h) volunteers were given the treatment with 200 ml of water. Mouth and tongue checks were performed to ensure the subject has ingested all of the medication. The volunteers were continuously monitored by West China Women's & Children's Hospital staff throughout the confinement period of the study. They were not permitted to lie down or sleep for the first 4 h after the treatment. Meals which had been fully inspected not to contain D-limonene were served 2, 4 and 8 h after the treatment. Blood samples, approximately 3 ml, were taken at 0 h (prior to treatment) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h after treatment. Samples were collected in heparinized glass tubes and centrifuged at 4000 rpm for 10 min. The plasma was separated and kept frozen at $-20\,^{\circ}C$ until analysis. The washout period was 7 days. Phase II and III were repeated in the same manner to complete the crossover design.

4.4. Sample preparation for GC-MS analysis

To a silanized glass tube containing 0.5 ml plasma, 20 µl of *n*-hexane, 20 µl of internal standard (naphthalane $1.528\ \mu g \cdot ml^{-1}$) and 1 ml of acetonitrile was added. The mixture was mixed for 3 min at a higher speed in a vortex mixer and then centrifuged at 4000 rpm for 10 min. The supernatant was transferred to another silanized glass tube containing 0.5 ml *n*-hexane. After that, the mixture was mixed for 5 min at a higher speed in a vortex mixer and centrifuged at 4000 rpm for 10 min. Finally, the supernatant was transferred into the GC autosampler vial, and 2 µl aliquot was injected into a GC-MS system.

4.5. GC-MS conditions

Plasma concentrations of D-limonene were determined with a validated gas chromatograph (GC) separation with mass spectrometric (MS) detection (Wang et al. 2007). All solvents used were of HPLC grade. D-Limonene standard was obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). The internal standard, naphthalane ($C_{10}H_{18}$), was purchased from the Shanghai Chemical Co., Inc. (Shanghai, China). Polygeline injection was bought from Wuhan Hualong Pharmaceutical Co., Ltd (Wuhan, China).

An Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass spectrometer, an Agilent Technologies 7683 series autosampler and Agilent ChemStation software (Agilent Technologies, Inc., USA) were used for the analysis. An HP-5 fused-silica capillary column [30 m (L) × 0.25 mm (ID) × 0.25 µm (thickness)] was used for separation. High purity helium was used as carrier gas at a flow-rate of $1.5\ ml \cdot min^{-1}$. The temperature of the injection port, interface and ion source were set at 270 °C, 280 °C, and 280 °C, respectively. The temperature program was set as follows: 60 °C for 1 min, and then increased to 140 °C at a rate of $20\,^{\circ}C \cdot min^{-1}$. The analytes were monitored by selected ion monitoring (SIM) mode. D-Limonene was detected at m/z of 93 with a retention time of 4.1 min, while the internal standard (naphthalane) was detected at m/z of 138 with a retention time of 4.4 min.

4.6. Pharmacokinetic analysis

Pharmacokinetic analysis was performed by means of a model independent method using PK software Drug and Statistics (Version 2.1.1, China). The maximum observed concentration (C_{max}) and the time of C_{max} (t_{max}) were experimentally obtained by observation. Elimination half-life ($t_{1/2}$), area under curve to last measurable concentration (AUC_{0-t}), and area under curve extrapolated to infinity ($AUC_{0-\infty}$) were software outputs.

4.7. Statistical analysis

Statistical evaluation was performed by Drug and Statistics software. ANOVA was performed to assess formulation, period, sequence and subject effects. The formulations were considered bioequivalent if geometric mean ratios (GMR) (percentage reference) of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max}, and their 90% confidence intervals (CI) were within 80–125% for natural log-transformed data. Pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} (relative to reference) were evaluated using the two one-sided tests procedure for natural logarithmic transformed data. Wilcoxon's signed rank test was used to compare t_{max}. All data analyses were conducted according to FDA recommendations for establishing bioequivalence (FDA 2001a).

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